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A common mechanism for protein cluster formation

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Polarized states on the membranes are characterized by focal accumulation of proteins and lipids at local concentrations far exceeding their levels typically found outside of these dense clusters. Principles of thermodynamics argue that formation and maintenance of such structures require continuous expenditure of cellular energy to combat the effect of molecular diffusion that relentlessly dissipates the clusters in favor of the spatially homogeneous state. Small GTPases are known to play a crucial role in the formation of several such polarized states. Their ability to consume stored energy and convert it into a potentially useful work by cyclically hydrolyzing GTP and coupling to various effectors in a nucleotide-dependent way, makes them eligible candidates to fulfill the requirements for the molecules involved in the mechanisms responsible for the maintenance of polarized states. Consistently, continuous nucleotide cycling of small GTPases has been found required for the emergence of structures in several well characterized cases. Despite this general awareness, the detailed molecular mechanisms remain largely unknown. In a recent study, not directly involving small GTPases, we proposed a mechanism explaining the emergence and maintenance of the stable cell-polarity landmark that manifests itself as a protein cluster positioned on the plasma membrane at the growing ends of fission yeast cells. Unexpectedly, this study has suggested a number of striking parallels with the mechanisms based on the activity of small GTPases. These findings highlight common design principles of cellular pattern-forming mechanisms that have been mixed and matched in various combinations in the course of evolution to achieve the same desired outcome—tightly controlled in space and time formation of dense protein clusters.

Key words: small GTPase, Cdc42, cell polarity, Tea1, Mod5, yeast, polarity landmark, pattern formation, mathematical modeling

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Fission yeast, with its nearly geometrically perfect spherocylindrical shape and highly regular dimensions, presents an example of a cell that normally grows exclusively at its hemispheric tips.[1] Polarized growth is maintained by the local activity of several small GTPases, of which Cdc42 plays a prominent role by directing exocytosis through both actin-dependent and actin-independent pathways.[2] While the clusters of activated Cdc42 appear to be self-sustained under the normal conditions, their precise localization at the opposite ends of the cell turned out to be specified by a separate mechanism.[3] An early genetic screen by Snell and Nurse[4] identified a mutant that in response to thermal stress exhibited a striking T-shaped growth pattern with a new growth zone formed in the middle of the cell. The responsible protein, identified as Tea1, was later shown to physically localize at the cell tips in the form of diffuse clusters with irregular particulate morphology.[5] The cause of polar localization was explained by the observation that Tea1 particles were found at the ends of microtubules that grow toward the cell tips. Depolymerization of these microtubules completely delocalized Tea1 from the tips. Later studies revealed a continuous plus-end-directed traffic of Tea1 particles transported along the growing microtubule bundles by the kinesin motor Tea2.[6] The impact of the growing bundle with the cell tip results in the eventual stall of the microtubule growth. The pause of the bundle end at the plasma membrane is followed

by inevitable depolymerization with the concomitant deposition of the Tea1 cargo onto the membrane. The mechanism of Tea1 association with the plasma membrane remained largely unknown until Snaith and Sawin[7] identified a peripheral membrane protein, Mod5, whose deletion resembled the phenotype of tea1Δ. Remarkably, in the absence of Mod5, Tea1 continued to be delivered by the microtubules, yet no appreciable accumulation of Tea1 was seen at the tips. Reciprocally, localization of Mod5 was found to depend on Tea1. While in the wild type cells Mod5 was preferentially co-localized with Tea1 at the cell tips, in tea1Δ cells Mod5 was uniformly distributed along the entire membrane. With the demonstration of the direct interaction between these two proteins,[8] Mod5 became firmly established as the membrane anchor for Tea1. Several additional proteins, including Bud6, Tea3, Tea4 and For3, have been found to associate with Tea1 at the cell tips and contribute to the control of polarized growth.[9] Tea1, however, remains the essential primary cluster component that defines the polarity landmark.

Customarily, whenever a protein is found localized in a spatially-concentrated focus, the major effort is invested in the identification of the mechanisms responsible for its localized delivery, such as polarized secretion or motor-mediated transport along the cytoskeletal elements. Alas, much less attention is given to the mechanisms that maintain the spatially-concentrated localization in the steady state. Ironically, the indisputable delivery role of microtubules has for a long time overshadowed the roles of other factors that together with the microtubule-mediated input determine the characteristic size, concentration profile and turnover of the Tea1 clusters. In most cases, a mere localized delivery is not sufficient to establish a polarized state since the homogenizing influence of thermal diffusion is a powerful force to be reckoned with. Indeed, a simple back of the envelope calculation shows that the cargo delivered by a single microtubule bundle, if permitted to diffuse freely on the membrane even with a modest diffusion coefficient 0.01 $\mu m^2/s$ typical of a transmembrane protein, in just 100 sec after the deposition will spread within the area with diameter 4 μm, the typical cross-section of a fission yeast cell. Given that the bundle-associated Tea1 packets arrive approximately once a minute,[10] the polarized delivery alone is not sufficient to account for the stable Tea1 cluster. To explain the existence of spatially-localized structures despite diffusive spreading, their components are often assumed to attach to either a pre-existing polymeric lattice, e.g., the acto-myosin cortex in mammals, or to be included in the de-novo formed polymer, e.g., clusters of death receptors in cells undergoing apoptosis[11] or glutamate receptors in the inhibitory synapses.[12] Without explicitly specifying the nature of lateral interactions within the Tea1 cluster, Snaith and Sawin[7] suggested that the Mod5-Tea1 interaction played a crucial role in promoting spatial focusing of the Tea1 polarity landmark.

We set out to test this conjecture and further extend our understanding of the principles underlying formation of the Tea1 polarity landmark by using both experimental and theoretical tools.[10] Mutual interdependence of Mod5 and Tea1 localization suggested a simple model in which Tea1 and Mod5 meet at the plasma membrane to form a stoichiometric complex that serves as a protomer for a polymeric coat-like structure. The resulting formation of a stable polymer follows the "diffusion-and-capture" mechanism introduced originally by Losick and colleagues[13] to describe formation of the spore coat in *Bacillus subtilis*. This mechanism implies that Tea1 and Mod5 should have equal mobilities within the cluster, which due to its irregular and rapidly evolving particulate morphology was termed by us "cluster-network." Surprisingly, comparison of the photobleaching experiments of the entire tip vs. its half demonstrated that this is not the case. While after bleaching the entire tip both Mod5 and Tea1 demonstrated equally slow fluorescence recovery, the half-tip photobleaching revealed that Mod5 was considerably more mobile than Tea1 that showed no difference between the 2 types of the FRAP setup. A similar behavior was observed within filopodia for the actin bundler fascin by Borisy and colleagues[14] who concluded that fascin remained highly mobile within filopodia while it showed little exchange with the cytoplasmic pool. The analogy between the behavior of Mod5 and fascin suggested that Mod5 readily interacted with immobile Tea1 resulting in the observed sequestration of Mod5 at the cell tips while remaining relatively mobile within the Tea1 cluster-networks. Furthermore, by varying the total amount of cellular Mod5 with regulated promoters, we found that the Tea1:Mod5 ratio within the cluster-network varied widely from 6:1 in the Mod5-dificient strain to nearly 1:1 in the Mod5 overproducer. Together with the FRAP findings, this result firmly rejected the simple stoichiometric complex model and prompted us to seek a more elaborate mechanism that could reconcile all experimental observations. If Mod5 does not play the role of the structural "glue" that holds the cluster-network together, why is it essential for its formation?

In search of an answer to this question, we proposed that the structural backbone of the polymer is provided by Mod5-independent Tea1-Tea1 "bonds" that could be either direct (as suggested by the earlier identified in vitro interaction) or mediated by other proteins. To ensure that thus formed polymer could uniformly cover 2D areas, we proposed that the local network connectivity, i.e., the maximal number of Tea1-Tea1 bonds formed by each Tea1 molecule, was at least 3. This requirement that originated from purely mathematical considerations received an unexpected experimental support. Indeed, Tea1 sequence analysis suggested the existence of a trimeric coiled-coil motif, whose deletion resulted in a mutant protein that continued to travel on microtubules but failed to accumulate at the cell tips. In the model, Mod5 could reversibly associate with either monomeric or polymeric Tea1 and insert/remove Tea1 monomers into/from the polymer by facilitating respectively formation or dissociation of the Tea1-Tea1 bonds. Remarkably, the proposed simple mechanism based on only 2 reversible biochemical interactions—the rapidly formed but unstable Tea1-Mod5 bond and stable, slowly formed Tea1-Tea1 bond—was sufficient to describe all existing experimental data as well as predict the results of genetic perturbations. As formulated, this mechanism bares similarities to

the vesicle coat assembly mechanisms that are based on the activity of small GTPases from the Arf family.[15,16] Indeed, both the membrane retention and incorporation of coatomers require binding to the GTPase. Also, from the analysis of FRAP experiments,[17,18] the coat lifetime is longer than that of the Arf-coatomer interaction, presumably allowing a single GTPase molecule to perform several rounds of coatomer recruitment-incorporation cycle during the formation of a single coat. Thus, the GTPase may effectively serve as a coat polymerization catalyst. The interaction between Mod5 and Tea1, however, may not be coupled to GTP hydrolysis, unlike the interaction between the GTPase and coatomers in the vesicle coats where such a link is a possibility. Instead, in our model, Mod5, which equally catalyzes the polymerization and depolymerization of the Tea1 cluster backbone, acts as a "plasticity factor" that ensures dynamic remodeling of the polymer. Acknowledging these differences, a parallel between the functional roles of Arfs and Mod5 still can be seen in that both are definitely required for the membrane tethering and possibly also for the incorporation of the monomers (coatomers or adaptors in the first case and Tea1 in the second) into the polymer. Both Arfs and Mod5 are the mobile elements of the respective polymeric structures that likely undergo several rounds of incorporation-detachment cycle during the life-time of the polymer. While cycling of Arf1 during the assembly of the COPI coats has not been uniformly supported by the literature,[15,16] more clear mechanistic justification has been provided for the dynamics of Sar1 GTPase during the assembly of COPII coats in which the coat components were shown to increase the rate of GTP hydrolysis on Sar1 and thus promote the recycling of the GTPase into the cytoplasm.[19]

Are the above similarities purely coincidental or indicative of some evolutionary relationship? Tea1 protein consists of 6 N-terminal kelch repeats that are expected to form a β-propeller[20] followed by an extensive C-terminus with multiple predicted coiled-coil motifs. Intriguingly, a protein fold consisting of a β-propeller and an α-solenoid folds has been characterized recently as an ancient ancestor for several nuclear pore complex and vesicle coat proteins including COPI, COPII and clathrin.[21,22] While these proteins are remarkably diverse in the way they interact with each other, all of them are capable of forming spatially extended polymeric lattices. In the case of vesicle coats, this process is often mediated by the small GTPases. Future identification of the Tea1 structure will shed light on a tantalizing possibility of Tea1 being one of the proteins of this structural class.

Perhaps yet deeper parallels between the mechanisms controlling the assembly of the Tea1 polarity landmark and those mediated by the small GTPases were suggested by the analysis of the microtubule bundles-Tea1 polarity landmark as a whole system. The comparison of the population-averaged concentration profile of Tea1 within the cell tips with a map of individual microtubule delivery events also registered in a population of cells (see Fig. 3E in the original work[10]) gave us a hint that some mechanism, which effectively prevents aberrant deposition events from "smearing" the Tea1 profile, might be in operation. Indeed, while the majority of deposition events fall within close proximity of the tip center, every now and then a microtubule bundle leaves its cargo far from the center or even entirely outside the hemispheric dome of the cell tip. One would expect that the steady state profile of Tea1 should be at least as wide as the profile of deposition events, or even wider given the inevitable action of molecular diffusion. Yet, the average Tea1 profile was found to reside well within the cell tip and align perfectly with its geometric center. In search for a potential explanation, we resorted to the analysis of our model that revealed a totally unexpected behavior. Indeed, simulating the de-novo formation of the landmark after re-polymerization of the microtubules, we found that, as Tea1 accumulated at the cell tip, the height-normalized Tea1 concentration profile became narrower rather than wider. This counterintuitive self-focusing behavior was limited by the effective diffusivity of the polymer and continued even after the total amount of Tea1 on the membrane ceased increasing. Thus, further polymerization at the cluster center was sustained at the expense of Tea1 removal from the cluster periphery. A remarkably similar self-focusing mechanism was proposed by Goryachev and Pokhilko[23] to explain the spontaneous formation of the cluster of activated Cdc42 in the budding yeast presumptive bud site.[24,25] The first major requirement for the existence of this mechanism, the autocatalytic recruitment and activation of Cdc42 in the cluster center, was explained by the molecular regulatory motif by means of which Cdc42 locally recruited its own activator, the Cdc24 GEF, through the mediation of the effector-GEF complex.[23,26] Second requirement was the continuous recycling of the cluster components upon deactivation of Cdc42 from the cluster periphery into the cytoplasm. The resulting cytoplasmic convective flux (see **Fig. 1A**) was shown to offset continuous diffusive spread of the cluster on the membrane. Moreover, the existence of a competitive cellular pool of cluster components that rapidly cycled between the cytoplasm and the membrane was proposed[23] and then experimentally shown[27] to be one of the mechanisms ensuring the uniqueness of the yeast bud.

While small GTPases are not involved in the formation of the Tea1 landmark, both requirements for the existence of self-focusing behavior are fulfilled by the proposed model of the Tea1-Mod5 interaction. Indeed, similar to the Arp2/3-mediated branched actin polymerization,[28] formation of the Tea1 network backbone is autocatalytic since the newly added monomers are postulated to form new nucleation centers. Thus, whether the network polymerizes or depolymerizes is determined by the Tea1 input intensity (microtubule-based delivery) and the local density of the polymer itself. Since both are low at the cluster periphery, this is there the depolymerization and recycling to the cytoplasm take place. At the same time, the center of the cluster, where the polymer attempts to grow, is automatically set to match the geometric center of the microtubule delivery events (compare **Fig. 1B**).[29,30] Unlike in the GTPase-based system, the energy necessary to maintain diffusion-counteracting self-focusing mechanism is consumed not within the cluster itself, but rather by the polymerizing microtubules (GTP) and molecular motors (ATP) that move along the

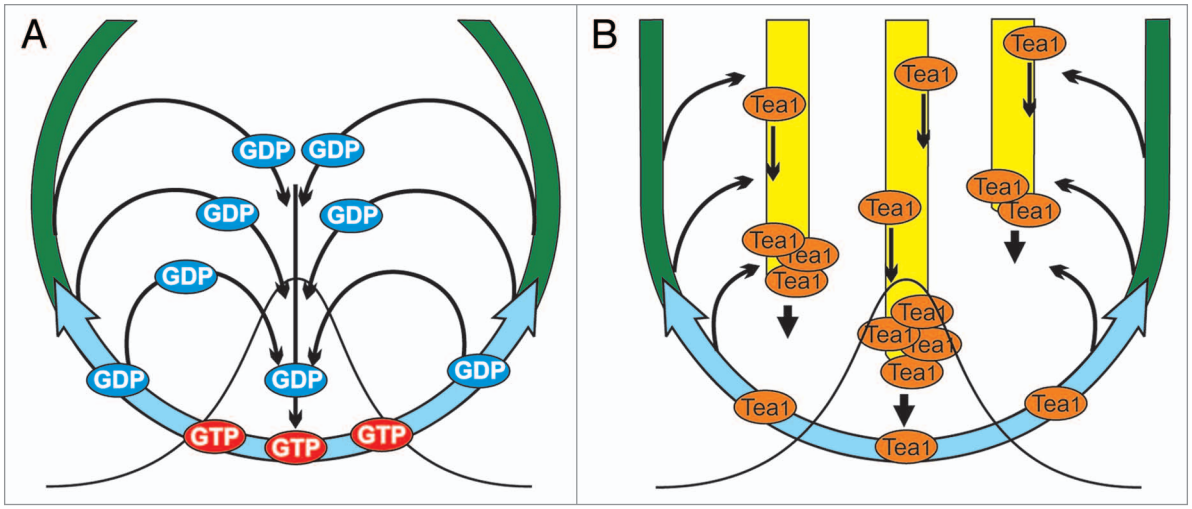

Figure 1. A class of membrane structures that are maintained in steady state by continuous convective cycling of their components. (A) Cdc42 is rapidly activated on the membrane in the center of the presumptive bud site due to the high local concentration of its GEF Cdc24. While diffusing along the membrane toward the periphery, Cdc42 is progressively deactivated and leaves the membrane. Nucleotide cycling of Cdc42 provides energy to drive continuous convective flux of Cdc42, its effectors and Cdc24. Vesicular traffic is not shown for simplicity. (B) Tea1 is delivered by microtubules onto the membrane at the cell tips, where, with the help of Mod5, it is partially incorporated into the Tea1 polymer. Mod5 that resides on the membrane and thus does not participate in the membrane-cytoplasmic cycle is not shown for simplicity. Drifting away polymer is depolymerized and Tea1 is recycled back to the cytoplasm to be captured by growing microtubules and re-enter the cycle. The convective flux of Tea1 is driven by the energy consumed by Tea2 kinesin motors (thin straight arrows) and by the polymerizing tubulin (thick short arrows). Bell-shaped curves represent symbolically the membrane concentration profiles of the activated Cdc42 (A) and Tea1 (B), respectively.

microtubules. Further theoretical and experimental work will undoubtfully provide other molecular realizations of the general cellular pattern-forming principles discussed here as well as reveal entirely distinct mechanisms permitting the generation of structures out of initially homogeneous starting conditions.

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